REMARKS

Entry of the foregoing, reexamination and reconsideration of the subject application, as amended, pursuant to and consistent with 37 C.F.R. §1.116, are respectfully requested in light of the remarks which follow.

I. Amendments to the Claims

By the foregoing amendments to the claims, claims 4-7 have been amended, claim 8 has been canceled, and new claims 13-15 have been added.

In particular, claims 4-5 have been amended to recite that the peptide is an "isolated" peptide. In addition, claim 7 has been amended by replacing the plural "diseases" with the singular "disease." These editorial amendments to the claims have been made to clarify the claim language and bring the claims into better conformance with U.S. patent practice, and are not intended to change the scope of the claims or any elements recited therein.

Claim 7 has been further amended by incorporating the subject matter of claim 8, with the exception of "capillarectasia."

New claims 13-15 depend from claim 7 and recite particular embodiments of the isolated peptide. Claims 13-15 are supported throughout the specification and claims as filed.

The amendments to the claims, including cancellation of claims, have been made without prejudice and disclaimer to any subject matter recited or canceled herein. Applicants reserve the right to file one or more continuation and/or divisional applications directed to any canceled subject matter. No new matter has been added, and entry of the foregoing amendments of the above-identified application are respectfully requested.

II. Information Disclosure Statement

The Examiner has returned an initialed and signed copy of the Form PTO-1449 that was submitted with the Information Disclosure Statement filed on July 17, 2007. All of the references listed have been considered, except for Kim et al. With regard to Kim et al., the Examiner has indicated that this reference was not considered because the copy provided with the July 17, 2007 Information Disclosure Statement was missing a number of pages.

In response, Applicants have resubmitted herewith a complete copy of Kim et al. Applicants respectfully request that the Examiner consider Kim et al., and return, with the next Office Action, an initialed and signed copy of the Form PTO-1449 indicating that the reference was considered.

III. Response to Claim Rejections Under 35 U.S.C. § 112, First Paragraph – Enablement

At page 3 of the Office Action, claims 7 and 8 have been rejected under 35 U.S.C. § 112, first paragraph, as purportedly lacking enablement.

Specifically, the Examiner has acknowledged that the specification enables the treatment of cancer, vascular malformation, arteriosclerosis, vascular adhesions, edematous sclerosis, corneal graft neovascularization, neovascular glaucoma, diabetic retinopathy, pterygium, retinal degeneration, retrolental fibroplasia, granular conjunctivitis, rheumatoid arthritis, systemic Lupus erythematosus, thyroiditis, psoriasis, pyogenic granuloma, seborrheic dermatitis and acne. However, the Examiner has stated that the specification does not enable treatment of capillarectasia or all other angiogenesis-related diseases.

In order to expedite prosecution in the present application, and not to acquiesce to the Examiner's rejection, the claims have been amended as described above. In particular, claim

7 has been amended to recite a method for the treatment of treatment of cancer, vascular malformation, arteriosclerosis, vascular adhesions, edematous sclerosis, corneal graft neovascularization, neovascular glaucoma, diabetic retinopathy, pterygium, retinal degeneration, retrolental fibroplasia, granular conjunctivitis, rheumatoid arthritis, systemic Lupus erythematosus, thyroiditis, psoriasis, pyogenic granuloma, seborrheic dermatitis and acne.

In addition, claim 8 has been canceled, rendering this rejection moot as to that claim.

Thus, the enablement requirement is met for the entire scope of the claims, and Applicants respectfully request reconsideration and withdrawal of this rejection.

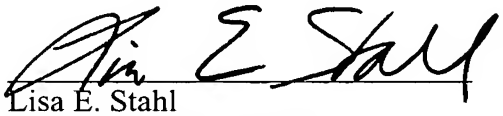
CONCLUSION

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited. In the event that there are any questions relating to this Amendment and Reply, or the application in general, it would be appreciated if the Examiner would telephone the undersigned attorney concerning such questions so that prosecution of this application may be expedited.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date: November 9, 2007

By: 

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RGD peptides released from βig-h3, a TGF-β-induced cell-adhesive molecule, mediate apoptosis

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βig-h3 is a transforming growth factor-β (TGF-β)-induced cell-adhesive molecule and has an RGD sequence at its C-terminus. A previous report suggested that βig-h3 normally undergoes carboxy-terminal processing that results in the loss of the RGD sequence. RGD peptides appear to play various roles in cell function. Here we show that the RGD peptides released from βig-h3 may facilitate TGF-β-induced apoptosis. We found that carboxy-terminal cleavage of βig-h3 occurred after its secretion, and that overexpression of the wild-type βig-h3 induced apoptosis, unlike the C-terminal deleted but RGD-containing mutant βig-h3, which is resistant to C-terminal processing. The βig-h3-induced apoptosis was abolished by either deletion of the RGD sequence or mutation of RGD to RAE. Synthetic peptides of ERGDEL and GRGDSP derived from βig-h3 and fibronectin, respectively, also induced apoptosis, unlike ERGEEL and GRGESP. Culture supernatants of cells overexpressing βig-h3 filtered to isolate molecules smaller than 3 kDa also induced apoptosis. A fusion protein composed of the N-terminal 100 amino acids of fibronectin and the RGD-containing C-terminal part of βig-h3 was also subjected to C-terminal cleavage and overexpression resulted in apoptosis. The anti-βig-h3 antibody blocks TGF-β-induced apoptosis. Thus, βig-h3 may be important in regulating cell apoptosis by providing soluble RGD peptides.
Oncogene (2003) **22**, 2045–2053. doi:10.1038/sj.onc.1206269

Keywords: βig-h3; RGD peptide; apoptosis; TGF-β; cell adhesion; anoikis

Introduction

βig-h3 is an extracellular matrix protein whose expression in several cell types, including human melanoma cells, mammary epithelial cells, keratinocytes, lung fibroblasts, and bone marrow stromal cells, can be induced by transforming growth factor-β (TGF-β) (Skonier *et al.*, 1994; Dieudonne *et al.*, 1999). The protein contains an RGD motif and four internal repeat domains that have highly conserved sequences also found in various secretory and membrane proteins from several species, including mammals, insects, sea urchins, plants, yeast, and bacteria (Kawamoto *et al.*, 1998). A number of studies have suggested that βig-h3 is involved in cell growth (Skonier *et al.*, 1994), cell differentiation (Dieudonne *et al.*, 1999; Kim *et al.*, 2000a), wound healing (Rawe *et al.*, 1997), and cell adhesion (LeBaron *et al.*, 1995; Ohno *et al.*, 1999). Although the underlying mechanisms driving these effects have not been defined, we recently reported that βig-h3 may mediate cell adhesion by interacting with the α3β1 integrin through two motifs residing within the 2nd and 4th repeat domains (Kim *et al.*, 2000b).

It has been reported that transfecting βig-h3-expressing plasmids into Chinese hamster ovary (CHO) cells led to a marked decrease in cell growth and a reduction in the ability of these cells to form tumors in nude mice (Skonier *et al.*, 1994). The same group reported that the recombinant βig-h3 expressed in the CHO cells did not retain its RGD sequence, an observation confirmed by carboxy-terminal sequencing. It was suggested that the loss was most likely because of carboxy-terminal processing. The RGD sequence was first discovered in fibronectin (FN) (Pierschbacher and Ruoslahti, 1984) and turned out to be a cell-attaching site found in many other adhesive proteins as well (Ruoslahti, 1996). The subsequent discovery of integrins, cell surface receptors that recognize the RGD sequence of various proteins, indicates that RGD plays a central role in cell adhesion biology. As integrin-mediated cell attachment influences and regulates cell migration, growth, differentiation, and apoptosis, RGD peptides and mimics have been used as probes to study such cell functions (Ruoslahti, 1996).

Whether endogenous soluble RGD-containing peptides released from matrix proteins are involved in the above-mentioned cellular functions is as yet not clear. With regard to apoptosis, however, soluble RGD peptides are known to induce a form of apoptosis denoted as 'anoikis'. Here, the RGD peptides compete

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with the cells for the extracellular matrix, thereby preventing the cells from adhering to the extracellular matrix and inducing them to apoptose (Frisch and Francis, 1994). A recent paper (Buckley *et al*., 1999) has also presented evidence for an alternative mechanism by which the RGD peptides facilitate apoptosis as they showed that a soluble RGD peptide directly activates a caspase in the cell cytoplasm. Supporting the putative role for RGD peptides in apoptosis, we have found that overexpressing the wild-type (WT) RGD-containing βig-h3 protein in CHO cells yields very few stable transfectants, whereas the overexpression of a mutant form whose RGD-containing C-terminal fragment does not get cleaved off yields many stable transfectants. Based on these preliminary observations, we hypothesize that the RGD peptide released from βig-h3 by carboxy-terminal processing might induce apoptosis. In this study, we show that βig-h3 indeed induces apoptosis by releasing RGD peptides.

Results

C-terminal cleavage of βig-h3 occurs after secretion

The expression plasmids encoding the wild-type βig-h3 (pMycβ-WT) and a mutant βig-h3 lacking its C-terminal 30 amino acids (pMycβ-ΔC30) are illustrated in Figure 1a. pMycβ-ΔC30 was constructed in the process of experiments on βig-h3 and unexpectedly, we found the following interesting result. Both plasmids express a protein with an Myc at its C-terminus. When transiently expressed in CHO cells, the pMycβ-ΔC30 protein, but not the pMycβ-WT protein, could be detected in the culture supernatant using the anti-*myc* antibody (Figure 1b). When the same samples were immunoblotted with the anti-βig-h3 antibody, both proteins were detected but the size and quantity of pMycβ-WT was less than that of pMycβ-ΔC30 (Figure 1c). In a parallel experiment, the culture supernatant from H1299 human lung carcinoma cells treated with TGF-β for 24 h was analysed on the same gel (Figure 1c). The size of the endogenous βig-h3 protein induced by TGF-β treatment was the same as that of pMycβ-WT. Thus, the WT βig-h3 undergoes C-terminal cleavage, which is consistent with the previously described observations (Skonier *et al*., 1994). Furthermore, the lack of its C-terminal 30 amino acids prevents it from being cleaved.

To determine whether WT βig-h3 cleavage occurs before or after secretion, we employed monensin to block protein secretion in CHO cells expressing the WT or deletion mutant βig-h3 proteins. When we used the anti-βig-h3 antibody, we detected proteins in the culture supernatants of both the monensin-treated and -untreated cells. Although monensin inhibited protein secretion but we could still detect βig-h3 in the culture

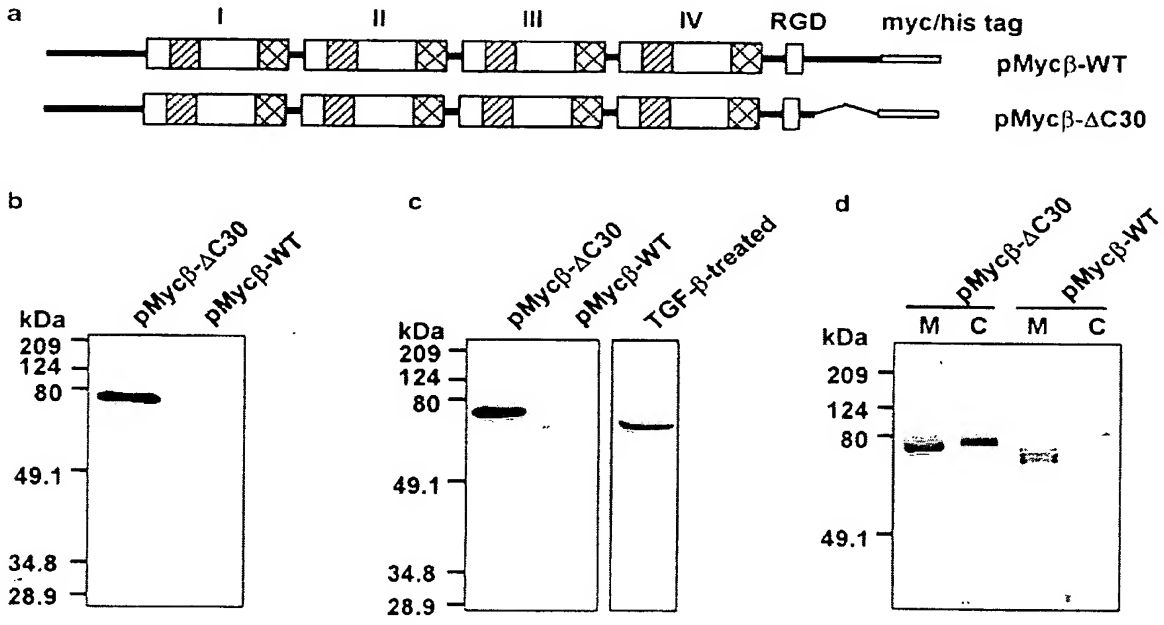

Figure 1 βig-h3 overexpressed in CHO cells undergoes C-terminal cleavage. (**a**) Mammalian expression vectors encoding the wild-type βig-h3 (pMycβ-WT) and the mutant βig-h3 lacking the C-terminal 30 amino acids (pMycβ-ΔC30). The hatched and crosshatched boxes indicate the highly conserved sequences in the repeat domains. The RGD motif is shown as an open box. The Myc epitope is attached at the C-terminus of each construct (**b**, **c**, **d**). Western blot analysis of pMycβ-WT and pMycβ-ΔC30 expressed in CHO by transient transfection. Proteins in the culture supernatants were subjected to 10% polyacrylamide gel electrophoresis and immunoblotted with anti-*myc*-HRP antibody (**b**) or anti-βig-h3 antibody (**c**). TGF-β-treated, H1229 cells were treated with 1 ng ml TGF-β for 24 h (**c**). CHO cells were transfected with pMycβ-WT or pMycβ-ΔC30 and then incubated with 0.5 μM monensin for 24 h. Samples from the culture media (M) and the cell layer (C) treated with monensin were immunoblotted with anti-βig-h3 antibody (**d**)

supernatant of monensin-treated cultures. However, proteins were detected in the cell layer only after monensin treatment (Figure 1d) as the untreated cells did not contain detectable levels of the βig-h3 protein (data not shown). The pMycβ-ΔC30 protein in the cell layer is slightly bigger than that in the culture supernatant. This was expected because βig-h3 is a secretory protein and has a signal peptide at its N-terminus that is retained in the cellular protein but not in the secreted protein. However, the WT βig-h3 in the cell layer is much bigger than either the WT βig-h3 or pMycβ-ΔC30 in the culture supernatant. These observations suggest that the cleavage of βig-h3 occurs following secretion.

The C-terminal fragment cleaved off the pMycβ-WT protein should include two epitopes, Myc and His, and is expected to be about 7 kDa. If this fragment is intact after being cleaved, it will be detectable after high percent gel electrophoresis of the cell supernatant. We used antibodies to both the Myc and His epitopes but failed to detect the fragment. This suggests that the fragment may undergo further digestion that results in smaller peptide fragments. Taken together, these results indicate that the C-terminal cleavage of βig-h3 occurs after it is secreted, resulting in the release of peptides.

Overexpression of βig-h3 induces apoptosis

Overexpression of βig-h3 is known to inhibit cell growth and thus we first investigated whether overexpressing βig-h3 induces apoptosis. We transiently transfected CHO and H1299 cells with the expression plasmids pMycβ-WT and pMycβ-ΔC30. A nucleosomal ladder appeared in the pMycβ-WT-transfected CHO and H1299 cells. In contrast, no DNA laddering was detected in cells transfected with either the vector or pMycβ-ΔC30 (Figure 2). That cells expressing WT βig-h3 undergo apoptosis was confirmed by propidium iodide staining. Furthermore, chromatin condensation and fragmented nuclei were observed in pMycβ-WT-transfected CHO and H1299 cells, but not in cells transfected with either the vector or pMycβ-ΔC30 (Figure 3). The βig-h3-mediated apoptosis was not confined to these two cell lines as it also induced

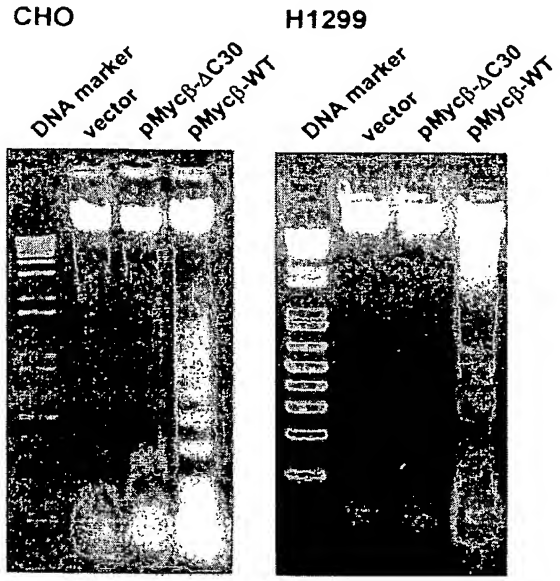

Figure 2 Overexpression of βig-h3 induces apoptosis in CHO and H1299 cells. DNA fragmentation in cells overexpressing pMycβ-WT or pMycβ-ΔC30 was assessed by agarose gel electrophoresis and ethidium bromide staining. The data shown are representative of three independent experiments

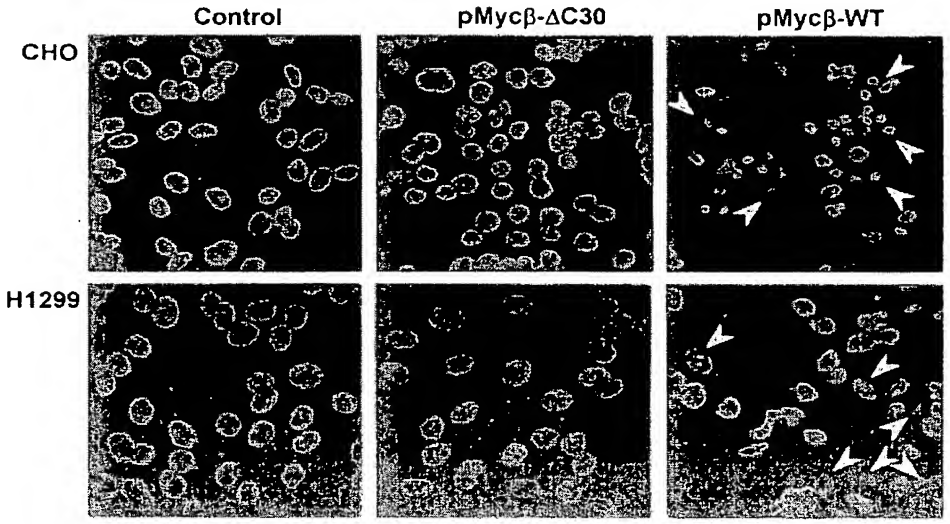

Figure 3 CHO and H1299 cells overexpressing βig-h3 develop fragmented nuclei. The nuclear morphology of cells transfected with the empty vector (Control), pMycβ-ΔC30, or pMycβ-WT was analysed as described under 'Materials and methods'. Data shown are representative of three independent experiments. Arrowheads indicate apoptotic bodies. Magnification, × 400

apoptosis in Hep3B hepatoma cells and HeLa cells (data not shown).

The RGD motif of βig-h3 is necessary for βig-h3-mediated apoptosis

We hypothesized that the RGD motif might play a key role in βig-h3-mediated apoptosis and tested this by generating two additional βig-h3 expression constructs (Figure 4a). pMycβ-ΔC44 expresses the βig-h3 protein lacking the C-terminal 44 amino acids, including the RGD motif, while pMycβ-RAE expresses the βig-h3 protein whose RGD motif has been mutated to RAE. That both mutants were expressed was confirmed by immunoblotting with the βig-h3 antibody (Figure 4b(b)). pMycβ-WT and pMycβ-RAE were not detected by the anti-*myc* antibody because of the C-terminal cleavage (Figure 4b(a)). Figure 4c reveals that deletion or mutation of the RGD motif completely abolished the apoptotic activity of βig-h3 in CHO and H1229 cells.

Synthetic peptide encoding a putative RGD peptide released by βig-h3 induces Apoptosis

To test whether exogenous RGD-containing peptides can directly induce apoptosis in CHO and H1229 cells, we used synthetic RGD peptides, one from βig-h3 (ERGDEL) and one from FN (GRGDSP), which was

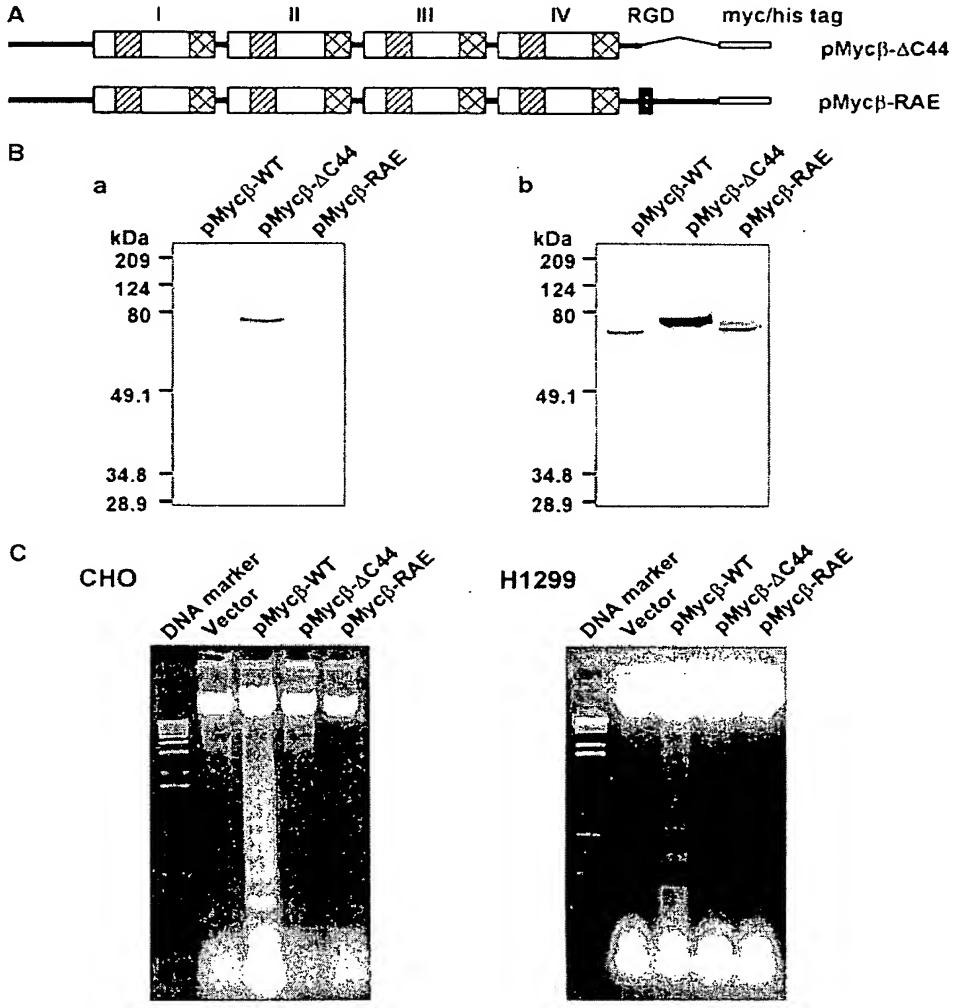

Figure 4 Deletion of the RGD motif or mutation of RGD to RAE abolishes βig-h3-mediated apoptosis. (a) Mammalian expression vectors encoding βig-h3 lacking the C-terminal 44 amino acids that include RGD (pMycβ-ΔC44) and βig-h3 whose RGD motif has been mutated to RAE (pMycβ-RAE). (b) Western blot analysis of βig-h3 proteins expressed by transiently transfected CHO cells. Proteins were subjected to 10% polyacrylamide gel electrophoresis and immunoblotted with anti-*myc*-HRP antibody (a) or anti-βig-h3 antibody (b). (c) DNA fragmentation in CHO and H1229 cells transfected with pMycβ-WT, pMycβ-ΔC44, or pMycβ-RAE was assessed by agarose gel electrophoresis and ethidium bromide staining. The data shown are representative of three independent experiments

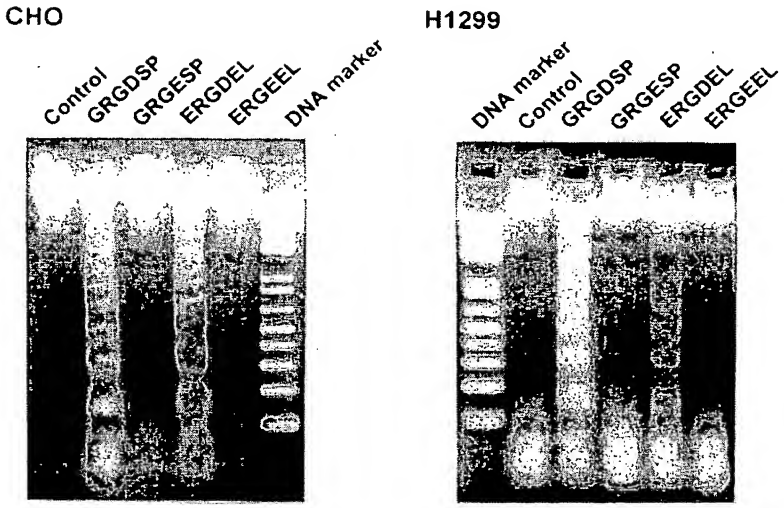

Figure 5 Synthetic RGD-bearing peptides induce CHO and H1229 cells to apoptose. DNA fragmentation in cells treated with 1 mM synthetic peptides for 2 days and in untreated cells was assessed by agarose gel electrophoresis and ethidium bromide staining. The data shown are representative of three independent experiments

used as a positive control peptide. Two negative control peptides, GRGESP and ERGEEL, were also employed. As shown in Figure 5, GRGDSP and ERGDEL clearly induced apoptosis in both cell lines whereas GRGESP and ERGEEL did not.

Culture supernatant putatively containing RGD peptides released from βig-h3 also induces apoptosis

To provide additional evidence to support the notion that RGD-containing peptides are released from cells expressing βig-h3 and that these peptides can mediate apoptosis, we collected culture supernatants from cells overexpressing WT or RAE mutant βig-h3 proteins. The supernatants were filtered through a membrane with 3 kDa molecular weight cutoff and the filtrate was added to CHO cells. The filtered culture supernatants from WT βig-h3-expressing cells induced apoptosis, whereas the filtered supernatants of control cells and cells expressing the RAE mutant did not (Figure 6). These observations are consistent with the notion that C-terminal peptides containing RGD are released from βig-h3 into the culture supernatant and they can induce apoptosis.

A fusion gene composed of the N-terminal part of FN and the C-terminal part of βig-h3 is also subjected to C-terminal cleavage and its overexpression induces apoptosis

To examine whether the expression of the C-terminal sequence of βig-h3 has a proapoptotic effect, we fused the C-terminal part of βig-h3 (amino acids 517–683) with the N-terminal 1.13 kb fragment of the human FN gene and cloned the resulting gene into the mammalian expression vector pcDNA (Figure 7a). CHO cells were then transfected. This fusion protein was not detected in

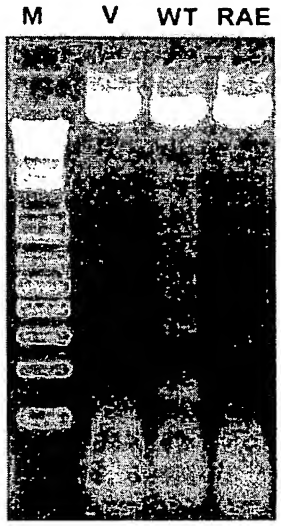

Figure 6 Culture supernatants of CHO cells overexpressing βig-h3 have apoptosis-inducing activity. Culture supernatants of CHO cells overexpressing WT or RAE mutant βig-h3 were filtered through a membrane with 3000 molecular weight cutoff and the filtrates added to CHO cell cultures for 20 h

the culture media by the anti-*myc* antibody but was observed in the monensin-treated cell layer (Figure 7b). This indicates that the secreted fusion protein undergoes C-terminal cleavage like βig-h3. We then tested whether expressing the fusion protein also induces apoptosis. As shown in Figure 7c, overexpression of WT βig-h3 and the fusion protein induced apoptosis, whereas the empty vector did not. Thus, it is possible that the C-terminal peptides released by the fusion protein, like those from βig-h3, have an apoptotic effect.

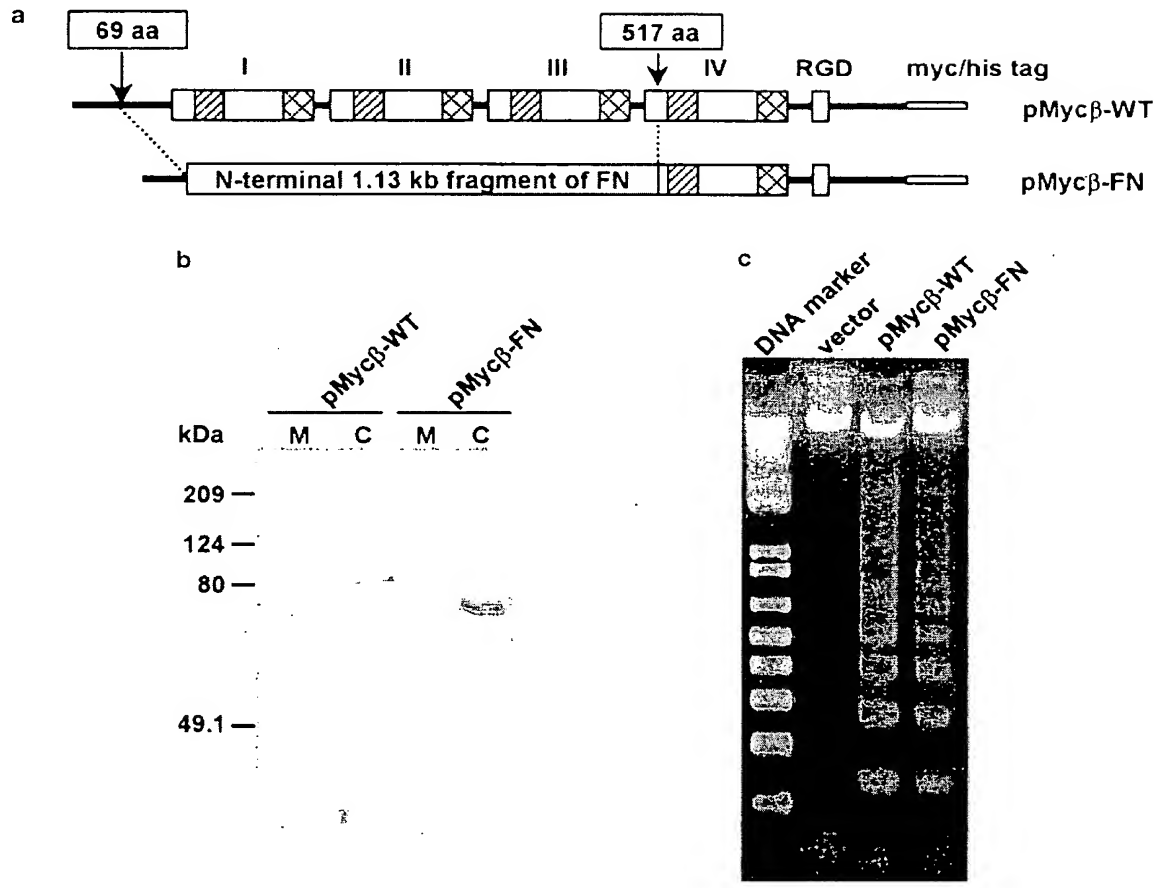

Figure 7 A fusion gene composed of FN and βig-h3 has apoptosis-inducing activity. (**a**) The mammalian expression vector encoding the WT βig-h3 (pMycβ-WT) and the fusion gene composed of the 100 N-terminal amino acids of FN and the C-terminal amino acids 518–683 of βig-h3 (pMycβ-FN). (**b**) Western blot analysis of CHO cells transiently transfected with pMycβ-WT or pMycβ-FN. Samples from the culture media (M) and the cell layer (C) treated with 0.5 μM monensin for 24 h were immunoblotted with anti-*myc*-HRP. (**c**) DNA fragmentation in CHO cells transfected with vector, pMycβ-WT, or pMycβ-FN was assessed by agarose gel electrophoresis and ethidium bromide staining

Anti-βig-h3 antibody blocks TGF-β-induced apoptosis

TGF-β is known to cause cells to apoptose as well as to induce the expression of βig-h3. To assess whether those two events are linked, namely, that βig-h3 expression induced by TGF-β treatment mediates the apoptotic effect of TGF-β, we treated H1229 cells with TGF-β in the presence of the anti-βig-h3 antibody. TGF-β-treatment for 2 days induced βig-h3 expression in a dose-dependent manner (Figure 8a). TGF-β also induced apoptosis of cells treated for 3 days. However, this apoptotic effect was significantly blocked by the presence of the anti-βig-h3 antibody treatment but not by control IgG (Figure 8b). This suggests that endogenously expressed βig-h3 is involved in TGF-β-induced apoptosis.

Discussion

In this study, we demonstrated that βig-h3 induces cells to undergo apoptosis through its RGD motif, which is released by the C-terminal cleavage upon secretion. It is known that many extracellular matrix proteins bear RGD sequences and other active cryptic sequences that are exposed as active soluble peptides. However, this usually occurs after the protein undergoes proteolytic fragmentation. As a TGF-β-induced cell adhesive molecule and being an extracellular matrix protein, βig-h3 may be unique in that shortly after its synthesis it releases its RGD peptide by C-terminal processing rather than full degradation. We could not show directly that RGD-containing peptides are released from βig-h3 in the culture supernatant as these peptides were too small for detection but the following observations indicate strongly that these peptides are generated and mediate apoptosis: (1) deletion or mutation of the RGD motif completely abolished the apoptotic activity of βig-h3; (2) ERGDEL, a synthetic RGD peptide derived from βig-h3, induced apoptosis whereas ERGEEL, a mutant peptide, did not; (3) molecules less than 3 kDa in size filtered from culture supernatants of cells overexpressing WT βig-h3 induced apoptosis; (4) the

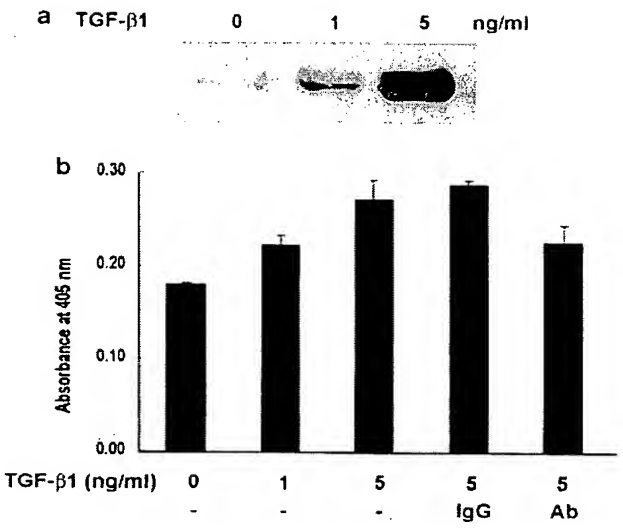

Figure 8 The anti-βig-h3 antibody blocks TGF-β-induced apoptosis. (a) βig-h3 protein induction by TGF-β1. H1229 cells were treated with the indicated concentrations of TGF-β1 for 2 days. βig-h3 protein in the culture supernatants was detected by the anti-βig-h3 antibody. (b) Effect of anti-βig-h3 antibody on TGF-β-treated H1229 cell apoptosis. H1229 cells were treated with TGF-β in the presence or absence of anti-βig-h3 antibody or control IgG protein for 3 days. Apoptotic cells were detected with the cell death detection ELISA kit. $*P<0.05$

overexpression of a fusion gene composed of the N-terminal 100 amino acids of the FN gene and the C-terminal part of the βig-h3 gene including the RGD sequence was also subjected to C-terminal cleavage and induced apoptosis.

It has generally been accepted that soluble RGD-containing peptides prevent integrin–ligand interactions, resulting in blocking the signals needed for cell survival (Frisch and Francis, 1994). The term 'anoikis' has been suggested to describe the apoptotic response to the absence of cell–matrix interactions mediated by soluble RGD peptides. Recently, Buckley *et al.* (1999) suggested that RGD peptides may activate the apoptotic cascade in a novel way, namely, by directly activating procaspase-3. Procaspase-3 has an RGD motif and a DDM sequence that resembles an RGD-binding site in integrin β-subunits and thus Buckley *et al.* theorized that RGD peptides trigger conformational changes, resulting in autoprocessing and the activation of procaspase-3. Supporting this possibility is a more recent report that an RGD peptide induces apoptosis in osteoblasts through caspase-3 (Perlot *et al.*, 2002). Thus, RGD peptides released from βig-h3 may induce apoptosis by directly activating caspase-3 and/or by disrupting cell–matrix interactions.

Given that βig-h3 is a secretory protein and that its RGD motif is released after secretion, it may be very difficult to accumulate a high enough local concentration of the RGD peptide to induce apoptosis *in vivo*. Such high local concentrations may occur only in certain environmental conditions, such as in tissues with few or no blood vessels. One such tissue is the cornea. Point mutations of the βig-h3 gene are responsible for the 5q31-linked corneal dystrophies (Munier *et al.*, 1997). These diseases are characterized by the progressive accumulation in the cornea of deposits containing βig-h3. This leads to epithelial cell degeneration and severe visual impairment. We have reported previously that five overexpressed mutant βig-h3 proteins frequently found in 5q31-linked corneal dystrophies also undergo C-terminal cleavage (Kim *et al.*, 2002). Thus, the accumulation of βig-h3 proteins in dystrophic corneas may increase the local concentration of RGD peptides, which then cause the degeneration of corneal epithelial cells.

The epiphysis of the long bones is also an avascular tissue. Interestingly, our preliminary experiments showed that the expression of βig-h3 in the long bone is localized to the prehypertrophic and hypertrophic chondrocytes of the epiphysis. It is well known that hypertrophic chondrocytes undergo normal cell death processing during endochondral bone formation. Therefore, we suggest that RGD peptides released from βig-h3 synthesized by prehypertrophic and hypertrophic chondrocytes may accumulate in the hypertrophic chondrocyte zone, thereby contributing to the apoptotic processing of hypertrophic chondrocytes during endochondral bone formation. RGD peptides released from βig-h3 may also be important in the normal apoptosis of the skin epidermis, which also does not have vessels. The keratinocytes of the granular layer epidermis express βig-h3 (LeBaron *et al.*, 1995) and eventually undergo apoptosis. These putative *in vivo* βig-h3 functions should be investigated, perhaps by constructing transgenic mice expressing βig-h3 in a tissue-specific manner such as in the chondrocytes of the long bone epiphysis or in the keratinocytes in the epidermis.

The expression of βig-h3 is highly induced by TGF-β treatment (Skonier *et al.*, 1992, 1994). TGF-β has been reported to induce apoptotic cell death in many cell types, although the mechanisms that regulate this process are still poorly understood. Previous studies suggest that the production of reactive oxygen intermediates (Sánchez *et al.*, 1996; Atfi *et al.*, 1997) and the activation of caspase-family proteases (Chen and Chang, 1997; Inayat-Hussain *et al.*, 1997) are involved in TGF-β-induced apoptosis. In addition, pRb (Choi *et al.*, 1999) and Bcl-2 (Lafon *et al.*, 1996) have been suggested to regulate TGF-β-induced apoptosis. More recently, it has been reported that TGF-β can generate apoptosis indirectly by inducing the expression of the connective tissue growth factor (Hishikawa *et al.*, 1999). Given that βig-h3 is induced by TGF-β and has apoptotic activity, it is possible that βig-h3 could also mediate TGF-β-induced apoptosis. Supporting this, we demonstrated in this study that TGF-β-induced apoptosis was reduced in the presence of the anti-βig-h3 antibody. However, not every cell type appears to be sensitive to apoptosis mediated by the TGF-β and the βig-h3 pathway. For example, fibroblasts, which constitutively produce high amounts of βig-h3, are not

sensitive to apoptosis mediated by TGF-β and βig-h3 (data not shown) whereas CHO cells, which do not produce βig-h3 even in the presence of TGF-β, are very sensitive to apoptosis mediated by TGF-β and βig-h3. Based on experiments with other cell lines, we found that cell lines producing βig-h3, endogenously in response to TGF-β are likely to be resistant to apoptosis mediated by TGF-β and βig-h3, whereas cell lines that do not produce βig-h3 even in the presence of TGF-β are likely to be sensitive (data not shown). Thus, sensitivity to apoptosis mediated by the TGF-β and the βig-h3 pathway depends on the cell type.

Of all the cell lines we tested, H1299 cell is the only cell line that produces βig-h3 and undergoes apoptosis in response to TGF-β. However, compared to most of the sensitive cell lines, H1229 requires higher TGF-β concentrations (5 *vs* 1 ng/ml) before it will produce βig-h3 and undergo TGF-β-induced apoptosis. This may explain why the apoptotic response of H1299 cells to TGF-β is so marginal.

Although cells producing βig-h3 in response to TGF-β may themselves not be significantly affected by βig-h3, neighboring cells may be more sensitive. TGF-β acts as a tumor suppressor by inhibiting cellular proliferation or by promoting cellular differentiation or apoptosis (Epstein, 2000). Although the role of βig-h3 in tumors has not been extensively studied, it has been reported to be downregulated in mesenchymal tumor cells (Schenker and Trueb, 1998) and rhabdomyosarcoma (Genini *et al*., 1996). Our observations suggest that the TGF-β-induced-βig-h3 could be a mechanism mediating TGF-β-mediated tumor suppression and that it may act as a tumor suppressor.

In summary, we demonstrated that upon secretion, βig-h3 produces RGD-containing peptides by C-terminal cleavage and that these peptides in turn induce apoptosis in several cell lines. βig-h3 is unique as an extracellular matrix protein as it generates soluble RGD peptides directly from the newly synthesized protein. RGD peptides can affect a variety of biological and pathological processes. Thus, by mediating cell adhesion and by providing soluble RGD peptides, βig-h3 may play important roles in regulating cell functions.

Materials and methods

DNA constructs

Full-length βig-h3 cDNA was subcloned into pcDNA3.1/Myc-His A (Invitrogen; Carlsbad, CA, USA) to generate the pMycβ-WT construct. The βig-h3 cDNAs lacking the C-terminal 30 and 44 amino acids were subcloned into the same vector, yielding the pMycβ-ΔC30 and -ΔC44 constructs, respectively. The substitution mutant of pMycβ-WT, where RGD was altered into RAE, was generated by a two-step PCR as described previously (Kim *et al*., 2000a). The 1.13 kb N-terminal fragment of human FN corresponding to amino acids 1–100 was subcloned into the *Kpn*I and *Sph*I sites of pMycβ-WT, thereby producing a fusion protein consisting of the FN N-terminal fragment fused to a C-terminal fragment of βig-h3 corresponding to amino acids 518–683. This construct was denoted pMycβ-FN.

Cell culture and transient transfection

CHO cells were cultured in α-MEM (Gibco BRL Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. H1299 are human lung carcinoma cells and were cultured in RPMI 1640 medium (Gibco) with 10% FBS, 4.5 g/l glucose, 10 mM HEPES, and 1 mM sodium pyruvate. For transient transfections, plasmids were transfected into cells using the lipofectamine reagent (Gibco) according to the manufacturer's protocol. For the secretion-blocking experiment, 0.5 μM monensin (Sigma) was added with the serum-free medium for 24 h. After incubation, the supernatants were lyophilized and analysed by immunoblot analysis using anti-βig-h3 antiserum made as described previously (Kim *et al*., 2002) and anti-*myc*-HRP antibody (Invitrogen). To isolate molecules smaller than 3 kDa from cell supernatants, the supernatants were filtered with Centricon YM-3 (Amicon, Millipore Corporation, Bedford, MA, USA) for 2 h. The filtrates were added to new CHO cells. Cells were harvested after 20 h and DNA was extracted for the DNA fragmentation assay. Western blotting was performed as described previously (Kim *et al*., 2000b).

DNA fragmentation assay

DNA samples were extracted by 30 min incubation at 0°C in 0.1 ml lysis buffer (100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 50 μg/ml freshly made proteinase K), recovered by isopropanol precipitation, resuspended in Tris-EDTA-RNase, analysed on 2% agarose gels, and visualized by ethidium bromide (1 μg/ml) staining.

Synthetic peptides

Synthetic peptides were synthesized on an automated multiple peptide synthesizer (PE/ABD 433, PE Corporation, Norwalk, CT, USA) using standard solid-phase procedures. Peptides were purified by reverse-phase high-performance liquid chromatography.

Assessment of apoptosis using propidium iodide

Cells collected after 2 days of incubation were washed twice with PBS, fixed in methanol : acetone (1 : 1) for 10 min, incubated for 1 h at 37°C with propidium iodide (50 μg/ml) in the presence of RNase A (10 μg/ml), and mounted in antifade reagent (Sigma). Immunofluorescence was analysed by a laser scanning confocal microscope (Axiovert 100; Lens 3 × 40/0.75; laser line 543, Carl Zeiss, Oberkochen, Germany). Pictures were taken on TMAX 100 film (Eastman Kodak Co., Rochester, NY, USA).

Assessment of apoptosis by ELISA

Cell apoptosis was assessed by the Cell Death Detection ELISA kit (Roche, Germany), which allows for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments. Briefly, cells collected after 3 days of incubation were washed and incubated in a lysis buffer for 30 min at room temperature. The lysates were then pelleted by centrifugation and the supernatant containing the cytoplasmic fraction was used for ELISA. The Student's *t*-test was used for statistical analysis and $P < 0.05$ was considered to be statistically significant.

Abbreviations

TGF-β, transforming growth factor-β; CHO cell, Chinese hamster ovary cell; FBS, fetal bovine serum.

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